

THE HUMAN NEUTROPHIL SERINE PROTEINASES,  
ELASTASE AND CATHEPSIN G, CAN MEDIATE  
GLOMERULAR INJURY IN VIVO

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Neutrophilic polymorphonuclear leukocytes (PMNs) have been implicated in the capillary wall injury in glomerulonephritis (GN) via release of proteinases and reactive oxygen species (1, 2). Although the acid proteinase cathepsin B can degrade isolated glomerular basement membrane (GBM) in vitro (3), most studies suggest that the major PMN proteinases that degrade GBM are the cationic neutral serine proteinases elastase and cathepsin G, and the neutral metalloproteinase gelatinase (3-5). Despite the impressive in vitro data supporting a role for PMN-derived proteinases in GBM injury, very little in vivo evidence of such a mechanism has been provided. We report here on the ability of elastase and cathepsin G to mediate glomerular injury in vivo.

Materials and Methods

**Reagents.** Elastase and cathepsin G were isolated from human PMNs (6) and assayed using the specific synthetic peptide substrates methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanalide (MSAAPV-NA) (Sigma Chemical Co., St. Louis, MO) and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma Chemical Co.), respectively. The specific activity of the elastase and cathepsin G was 130 and 36 U/μg protein, respectively, where 1 U caused a change in absorbance of 0.001/min at 400 nm and 25°C. Neither the cathepsin G nor elastase substrate reacted with the other enzyme preparation. In some experiments, elastase and cathepsin G were irreversibly inactivated with the specific chloromethyl-ketone inhibitors MSAAPV-chloromethyl ketone (MSAAPV-CK) (Sigma Chemical Co.) and Z-Gly-Leu-Phe-chloromethyl ketone (Enzyme Systems Products, Livermore, CA). Elastase and inactivated elastase were equivalently cationic (pI > 10.5) by isoelectric focusing.

**Renal Artery Perfusion.** Sprague-Dawley rats were anesthetized with chloral hydrate, and a left nephrectomy was performed. The aorta was clamped and the right renal artery was perfused at a flow rate of 0.5 ml/min as described (7). Initially, 0.5 ml PBS was perfused to displace blood from the kidney. Rats were then perfused with 50 μg of active (*n* = 13) or inactivated elastase (*n* = 6) in 0.5 ml PBS. Control rats received PBS alone (*n* = 15).

These studies were presented in part at the 20th Annual Meeting of the American Society of Nephrology in Washington D.C., December 1987, and have been published in abstract form (*Kidney Int.* 33:331, 1988). Address reprint requests to Dr. Richard J. Johnson, M.D. Division of Nephrology, RM-11, University of Washington, Seattle, WA 98195. This work was supported by U.S. Public Health Service grants AL-07763, DK-34198, DK-39068, and research grants from the Northwest Kidney Foundation.

Additional rats ( $n = 19$ ) were perfused with smaller amounts of active elastase (1–25  $\mu\text{g}$ ) to establish a dose-response relationship, and four rats received 1 mg of cationized IgG (pI 8.1–> 9.3) (8). Finally, some rats received either active ( $n = 8$ ) or inactivated cathepsin G ( $n = 4$ ). After an additional 4 min, the kidney was cleared with 0.3 ml of PBS, and blood flow was restored. Only kidneys that reperfused normally were included in the study, and total ischemia time was always <8 min. After recovery from anesthesia, the rat was placed in a metabolic cage with free access to water.

**Localization of Elastase.** In separate experiments, elastase was localized by immunofluorescence using anti-elastase antibody raised in New Zealand rabbits. Elastase ( $n = 3$ ), or inactive elastase ( $n = 3$ ) (dose; 50  $\mu\text{g}$ ) was perfused as described above, but was followed by 0.5 ml PBS and then 1 ml of PBS containing 40 mg of anti-elastase rabbit IgG. Control rats received elastase followed by 40 mg of normal rabbit IgG ( $n = 2$ ) or PBS followed by anti-elastase antibody ( $n = 2$ ). 10 min after reperfusion, the kidney was biopsied and sections were stained for rabbit IgG with FITC-conjugated goat anti-rabbit IgG (Cappel Laboratories, Malvern, PA) as described (7).

To quantify the binding of elastase to the glomerulus, rats were perfused with 50  $\mu\text{g}$  trace-labeled  $^{125}\text{I}$ -elastase ( $n = 4$ ) or  $^{125}\text{I}$ -inactive elastase ( $n = 4$ ) as described above, followed by 9 ml of PBS to remove unbound  $^{125}\text{I}$ . The elastase, labeled with  $^{125}\text{I}$  by the chloramine T method, remained enzymatically active. The kidney was removed, the glomeruli isolated (7), and the uptake of  $^{125}\text{I}$  measured with a gamma counter. The total amount of elastase bound per glomerulus was calculated from the specific activity of the injected active or inactive elastase. For comparison, the amount of elastase released by human PMNs ( $10^7/\text{ml}$ ) was measured after a 2-min preincubation with 5  $\mu\text{g}/\text{ml}$  cytochalasin B (Aldrich Chemical Co., Milwaukee, WI) followed by a 20-min incubation with  $10^{-7}$  M FMLP (Peninsula Laboratories, Inc., Belmont, CA) ( $n = 4$ ). Cells were centrifuged at 1,000  $g$  for 5 min, and the supernatant was assayed for elastase activity.

**Morphologic Studies.** After perfusion with 50  $\mu\text{g}$  of active or inactive elastase, kidney biopsies ( $n = 30$ ) were performed at 10 min, 2, 24, and 48 h. Two rats that received elastase were also biopsied at 4 and 9 d. No individual rat had more than three biopsies. Kidney biopsies were also performed on all rats perfused with active or inactive cathepsin G 24 h after perfusion. Tissue for light and electron microscopy was processed as described (7), and sections for light microscopy were stained with periodic acid Schiff reagent.

**Statistical Analysis.** All data shown are mean  $\pm$  SE. Analysis between groups was performed using the Student's  $t$  test.

## Results

**Proteinuria After Elastase Perfusion.** The infusion of 50  $\mu\text{g}$  of active elastase into the renal artery of rats resulted in massive proteinuria that was not seen in control rats that received inactive elastase or PBS alone (Table I). In most rats, the proteinuria resolved in 2–3 d, but in three rats, it persisted for 6–9 d (i.e., >40 mg/24 h). Four rats perfused with large doses (i.e., 1 mg) of cationized human IgG failed to develop the degree of proteinuria that was observed in the elastase-perfused animals (Table I). In most studies, the elastase was allowed to remain in the ischemic kidney for 4 min before reperfusion. However, three elastase-perfused rats, in whom the blood flow was immediately restored, developed impressive proteinuria in the initial 24 h after surgery ( $163 \pm 57$  mg/24 h), suggesting that the delay in reperfusion was not essential for injury. A linear relationship between the dose of elastase infused and the degree of proteinuria was noted (no elastase,  $15 \pm 3$  mg/24 h,  $n = 15$ ; 5  $\mu\text{g}$  elastase, 3 mg/24 h,  $n = 2$ ; 10  $\mu\text{g}$  elastase,  $33 \pm 4$  mg,  $n = 4$ ; 15  $\mu\text{g}$  elastase,  $40 \pm 5$  mg,  $n = 5$ ; 20  $\mu\text{g}$  elastase,  $82 \pm 51$  mg,  $n = 4$ ; 25  $\mu\text{g}$  elastase,  $77 \pm 22$  mg,  $n = 4$ ; 50  $\mu\text{g}$  elastase,  $196 \pm 32$  mg,  $n = 13$ ;  $r = 0.7$  by linear regression,  $p < 0.001$ ).

TABLE I  
*Urinary Protein Excretion (mg/24 h) after Renal Artery  
 Perfusion of Various Perfusates*

Perfusate	Day 0-1		Day 1-2	
	Mean ± SE	n	Mean ± SE	n
Elastase (50 µg)	196 ± 32	(13)*	42 ± 12 <sup>†</sup>	(8)
Inactive elastase (50 µg)	19 ± 2	(6)	8 ± 1	(6)
PBS	15 ± 3	(15)	ND	
Cationized IgG (1 mg)	25 ± 11	(4)	12 ± 3	(3)

Values are mean ± SE.

\*  $p < 0.005$ , relative to other groups.

<sup>†</sup>  $p < 0.05$ , relative to other groups.

The threshold dose of elastase that was necessary to induce significant proteinuria was 10–15 µg.

*Glomerular Localization of Elastase.* The infused elastase was localized in the glomerulus by fluorescent staining of a rabbit antibody to elastase administered in vivo (Fig. 1 *A*). Inactivated elastase, which has a similar isoelectric point, also localized to the glomerular capillary wall (Fig. 1 *B*). Neither control rats receiving elastase followed by normal rabbit IgG nor rats given PBS and anti-elastase antibody had any detectable rabbit IgG in their glomeruli. The infusion of 50 µg of <sup>125</sup>I-elastase or <sup>125</sup>I-inactive elastase resulted in comparable binding to glomeruli (324 ± 82 pg/glomerulus vs. 318 ± 67 pg/glomerulus,  $p = \text{NS}$ ), indicating that inactivation of elastase did not affect the degree of glomerular binding. Human PMNs pretreated with cytochalasin B released 3.3 µg elastase/10<sup>6</sup> PMNs on incubation with 10<sup>-7</sup> M f-met-leu-phe. Given our observation that the renal artery perfusion of 50 µg elastase results in the binding of 324 pg elastase/glomerulus, it can be calculated that the amount of elastase that is present in an individual glomerulus in rats perfused with 50 µg elastase could theoretically be provided locally by 100 human PMNs.

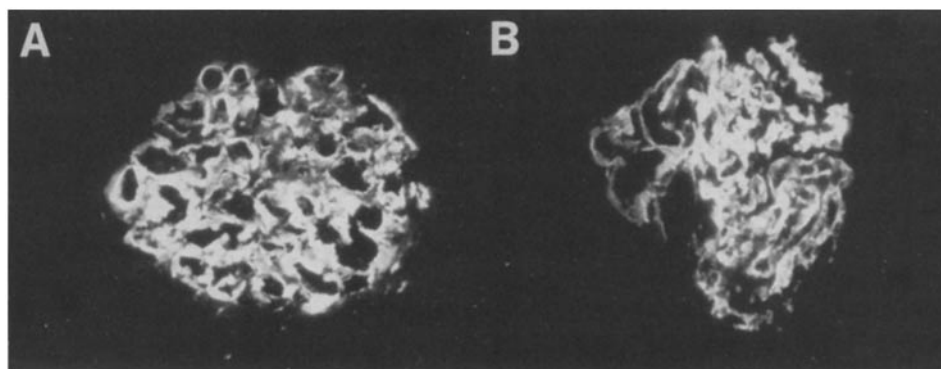


FIGURE 1. The infusion of elastase (*A*) or inactive elastase (*B*) into the renal artery of rats results in its localization to the glomerular capillary wall as demonstrated by immunofluorescence using a rabbit antibody to elastase ( $\times 400$ ).

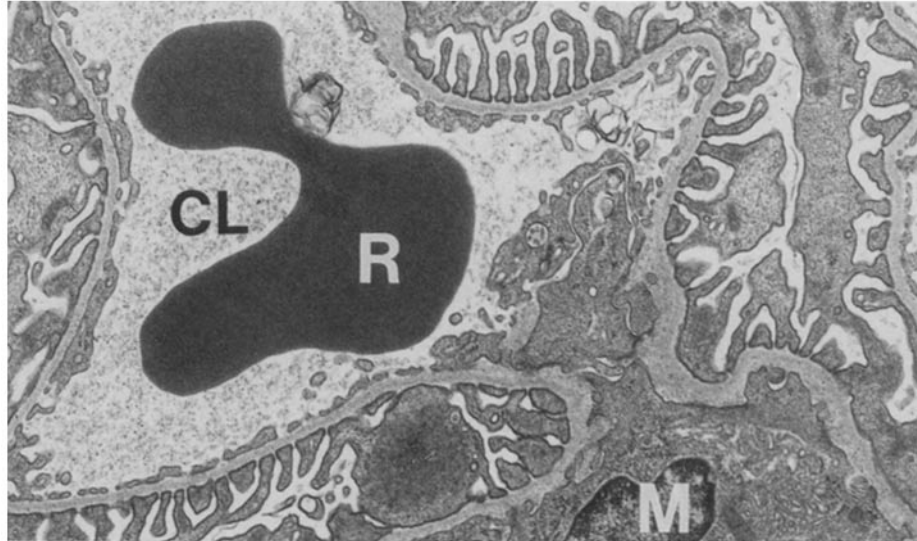


FIGURE 2. Glomerulus of a rat 24 h after the renal artery was perfused with 50  $\mu$ g of active elastase. The endothelium is intact, the GBM is of normal thickness, and there is preservation of epithelial cell foot processes. No inflammatory cells are evident. (CL, capillary lumen; M, mesangial cell; R, red blood cell) ( $\times$  18,500).

*Morphology of Elastase-perfused Rats.* Light microscopy was performed on elastase and inactive elastase-perfused rats at multiple times after perfusion (10 min, 2, 24, 48 h, 4, and 9 d). At no time was proliferation of resident glomerular cells or infiltration by blood-borne inflammatory cells seen. The GBM was always of normal thickness and did not display any gaps or splitting. At day 9, there were occasional glomeruli that had focal mesangial areas with decreased nuclear staining.

EM was performed on elastase-perfused rats at 10 min, 2, 24 (Fig. 2), and 48 h, and on days 4 and 9. No increase in mesangial matrix or cell number was noted. Glomerular endothelial cells displayed no evidence of injury or proliferation, and the GBM appeared normal. Epithelial cell foot processes remained largely intact with only focal areas of fusion noted. There was no evidence of neutrophil, monocyte, or platelet infiltration, and fibrin deposition was absent.

*Effect of Cathepsin G Infusion.* The renal artery perfusion of 50  $\mu$ g of active cathepsin G was associated with marked proteinuria that was not observed in control rats receiving inactivated cathepsin G ( $228 \pm 25$  mg vs.  $23 \pm 2$  mg/24 h,  $p < 0.001$ ). The administration of 5  $\mu$ g of cathepsin G did not induce proteinuria ( $24 \pm 3$  mg/24 h,  $n = 4$ ). Light microscopy performed on cathepsin G perfused rats at 24 h ( $n = 4$ ) was not different from the controls receiving inactive cathepsin G ( $n = 4$ ). There was no proliferation, and only an occasional PMN was observed. The GBM was of normal thickness and without gaps or splitting. EM was not performed.

### Discussion

These studies provide the first direct evidence that the human PMN serine proteinases, elastase and cathepsin G, can mediate GBM injury and proteinuria in vivo.

The infusion of elastase resulted in its localization to the glomerular capillary walls possibly via charge interactions between elastase, which is cationic ( $pI > 10$ ), and the rich anionic sites present in GBM. Quantitative binding studies demonstrated that the amount of elastase bound per glomerulus after perfusion of a proteinuric dose of 20  $\mu\text{g}$  could be provided by 40 human PMNs. The average number of PMNs per glomerulus in human GN varies greatly, but in certain diseases may be as many as 17 per 6  $\mu\text{m}$  glomerular cross-section (9). Therefore, it is likely that the amount of elastase (and possibly cathepsin G) bound to our rat glomeruli could have been released locally by PMNs in human GN. These estimates are approximate, since PMNs attracted to glomeruli in vivo would not be expected to release all of their elastase or cathepsin G. However, the concentration of the proteinases at the site of degranulation (i.e., in the space between an adherent PMN and GBM) would be expected to be high. In addition, proteinases released into this microenvironment may be protected from plasma inhibitors (e.g.,  $\alpha$ -1-proteinase inhibitor and  $\alpha$ -2-macroglobulin) (10). In our studies, inactivation by plasma inhibitors was minimized by perfusion into a bloodless kidney.

The observation that the proteinuria in our studies was in the nephrotic range is highly suggestive of an injury to the glomerular capillary wall resulting in an increase in its permeability. It is of interest, therefore, that the glomeruli appeared to be normal histologically. Even epithelial cell foot process fusion, which is observed in almost all severely proteinuric states, was absent. In contrast, the perfusion of the isolated components of the myeloperoxidase (MPO)- $\text{H}_2\text{O}_2$ -halide system into the renal artery results in proteinuria and marked histologic injury characterized initially by endothelial cell damage, platelet influx, and focal epithelial cell foot process fusion, and later (i.e., 4–10 d) by a proliferation of resident glomerular cells (11, 12). Since the release by the PMN of both proteinases and components of the MPO system would be anticipated within the glomeruli in PMN-dependent GN, it is likely that both would contribute to the injury observed, with the proteinases primarily causing changes in permeability and the MPO-derived oxidants producing changes in permeability and also mediating cellular damage.

We do not believe that the proteinuria observed in our elastase-perfused rats was secondary to charge neutralization of the glomerular capillary wall. The infusion of much larger (i.e., 20 times) quantities of cationized IgG did not reproduce the degree of proteinuria, nor did inactive elastase, although the latter is as cationic as native elastase. The fact that the proteinuria could not be reproduced by inactivated proteinases strongly suggests that the observed effects were due to proteolytic activity. The mechanism responsible for the proteinuria most likely involves degradation of basement membrane. Elastase and cathepsin G can degrade many basement membrane proteins, including type IV collagen, fibronectin, and the protein core of various proteoglycans (13). Although these proteinases can also injure or affect the function of various cell types (13), we did not observe any histologic evidence for endothelial cell injury or inflammatory cell involvement.

### Summary

We infused microgram quantities of active or inactive PMN elastase and cathepsin G into the renal arteries of rats. Both active and inactive elastase localized to the glomerular capillary wall equally, and in amounts that could be achieved physiologi-

cally in GN. However, elastase-perfused rats developed marked proteinuria ( $196 \pm 32$  mg/24 h) compared with control rats receiving inactive elastase ( $19 \pm 2$  mg/24 h,  $p < 0.005$ ). Similar results were seen with active and inactive cathepsin G. Neither elastase nor cathepsin G infusion was associated with histologic evidence of glomerular injury. We conclude that the PMN neutral serine proteinases elastase and cathepsin G can mediate marked changes in glomerular permeability in vivo due to their proteolytic activity, and thus, may contribute to the proteinuria observed in PMN-dependent models of GN.

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